RESEARCH ARTICLE

Colletotrichum fructicola, a Member of Colletotrichum gloeosporioides sensu lato, is the Causal Agent of Anthracnose and Soft Rot in Avocado Fruits cv. "Hass"

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ABSTRACT

The filamentous Ascomycota Colletotrichum gloeosporioides sensu lato is a fungus that has been reported worldwide as a causal agent of anthracnose disease in avocado and other crops. In Mexico, this species affects fruits from an early stage of development in the orchard until the post-harvest stage. Although fungicides are continuously applied to control Colletotrichum species, pericarp cankers and soft rot mesocarp in fruits are still frequently observed. Considering the lack of a precise description of the causative agent, the aim of the current study was to determine the pathogens involved in this symptomatology. Twenty-four isolates were consistently obtained from the pericarp of avocado fruits cv. "Hass" collected in the central avocado-producing area of Mexico. Morphological features such as colony growth, conidia size, and mycelial appressorium were assessed. Bayesian multilocus phylogenetic analyses were performed using amplified sequences of the internal transcribed spacer region of the nuclear ribosomal DNA; actin, chitin synthase, glyceraldehyde-3-phosphate dehydrogenase partial genes; and APn2-Mat1-2 intergenic spacer and mating type Mat1-2 partial gene from the nine selected isolates. In addition, fruits were inoculated with a conidial suspension and reproducible symptoms confirmed the presence of Colletotrichum fructicola in this area. This pathogenic species can now be added to those previously reported in the country, such as C. acutatum, C. boninense, C. godetiae, C. gloeosporioides, and C. karstii. Disease management programs to reduce the incidence of anthracnose should include C. fructicola to determine its response to fungicides that are routinely applied, considering that the appearance of new species is affecting the commercial quality of the fruits and shifting the original population structure.

1. Introduction

Anthracnose is a disease caused by the Colletotrichum species complex, an intracellular filamentous Ascomycota that is characterized by the formation of sunken spots and canker in the stems, twigs, branches, and fruits [1]. This pathogen affects many hosts worldwide such as avocado (Persea americana), annona (Annona cherimola), coffee (Coffea arabica), apple (Malus domestica), peach (Prunus persica), mango (Mangifera indica), and, more recently, tea oil (Camellia oleifera), among others [2–6].

Colletotrichum gloeosporioides has been extensively studied, and a species complex comprising 22 species along with one subspecies using multilocus phylogenetic analysis was recently described [7], and shown to be formed by 42 accepted species [8–10].

This species complex has been reported as the causal agent of anthracnose in all avocado-producing countries, and represents the most serious post-harvest disease in high rainfall growing regions [11]. Furthermore, avocado anthracnose has been reported to be caused by C. gloeosporioides in South Africa [12] and Israel [13], C. fioriniae in Australia [14], C. aenigma in Israel, C. alienum in Australia and New Zealand, C. fructicola in Australia [7], C. kahawae subsp. ciggaro in New Zealand, C. queenslandicum in Australia, and C. siamense in Australia and South Africa [7].

In Mexico, C. gloeosporioides, C. boninense, C. acutatum [15], C. godetiae [16], and C. karstii [17] have been isolated from avocado exocarp tissue with anthracnose symptoms. Nevertheless, there is no mention of C. gloeosporioides sensu stricto causing anthracnose in avocado in Mexico, which may

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be because previous studies only assessed *C. gloeosporioides* using a single locus based on the internal transcribed spacer (ITS) region of rDNA, with no comparison to newly redefined species belonging to *C. gloeosporioides sensu lato*. Indeed, the ITS region was proposed as a fungal barcode marker [18] and has been used for *Colletotrichum* identification in several studies [15,19–21].

Nevertheless, multilocus phylogenetic analyses have clarified that the species included in the *C. gloeosporioides* complex are plant pathogenic fungi [7,22]. Therefore, the precise identification of phytopathogenic species of *C. gloeosporioides* that cause necrosis in the pericarp tissue and the soft rot mesocarp of fruits is very important [23]. This would allow for the rational and adequate use of fungicides to reduce the incidence and severity of disease [24], and provide the knowledge required for determining the sources of the primary inoculum from which the infection cycle begins in fruits [1].

Here, we demonstrate that anthracnose symptoms are present in unripe avocado fruits in the field in the central part of Mexico, and provide accurate identification of the associated causal agents using morphological and phylogenetic approaches.

2. Material and methods

2.1. Sampling and fungal isolation

In 2015, isolates of *Colletotrichum* were obtained from fruits recovered in an orchard located in Molango de Escamilla, state of Hidalgo, Mexico at 1663 m above sea level. Thirty avocado fruits cv. "Hass" exhibiting anthracnose symptoms in the pericarp were sampled for fungal isolation. These symptoms are represented by the formation of cankers as small circular necrotic areas on the fruits. Exocarp tissue fragments, taken from the canker advance zone, were washed in tap water, surface-disinfested in a 1.5% sodium hypochlorite solution for 2 min, and rinsed three times with sterile distilled water (SDW). Small pieces of ~5 mm^2 were dried in a laminar flow chamber on sterile paper towels and placed onto Petri plates containing potato dextrose agar (PDA) medium (Difco, NJ, USA). The plates were incubated at 28 °C in the dark for five days, and then fungal colonies were transferred to PDA plates and maintained at 28 °C in the dark until conidia were observed.

To obtain single conidium isolates, the conidia were harvested by adding 5 mL of SDW to the plates, and the concentration of the suspension was adjusted to 1×10^6 conidia/mL using an hemocytometer (Marienfeld, Germany). A 100-μL aliquot was spread on water agar and incubated at the same temperature as above in the dark for 24 h. A single

germinating conidium was transferred to a fresh PDA plate. Twenty-four isolates were obtained and morphologically identified as *Colletotrichum* based on morphological features of the genus.

2.2. Pathogenicity test

Mycelial discs from a five-day-old actively growing edge colony on PDA medium were transferred to oatmeal agar (OA: 20 g oatmeal, 20 g agar, 1 L distilled water) plates, and scraped onto the surface of the medium to induce sporulation [25]. The plates were incubated at room temperature (24–26 °C) for 10 days.

Twenty-five healthy unripe avocado fruits cv. "Hass" were washed with running tap water and surface-disinfested in 1.5% sodium hypochlorite for 5 min, rinsed three times with SDW, and then dried at room temperature. A conidial suspension (10^6 conidia/mL) was prepared from each 10-day-old monoconidial *Colletotrichum* culture by adding 5 mL of SDW to an OA plate. The fruits were inoculated using the non-wound drop inoculation method. Thirty microliters of the conidial suspension was placed on the middle of each fruit. Control fruits were inoculated with 30 μL of SDW. The inoculated samples were incubated in containers at room temperature in normal light for 10 days [26]. Subsequently, the fruits were cut to observe the development of the typical soft rot lesions in the mesocarp. The fungus was re-isolated from the inoculated fruits to fulfill Koch's postulates, and the identity was determined using a morphological approach.

2.3. DNA extraction, polymerase chain reaction (PCR) amplification, and sequencing

For each *Colletotrichum* isolate, a small amount of mycelium was scraped from the surface of a 5-day-old culture (grown on PDA at 28 °C) using a sterile 10-μL pipette tip, and genomic DNA was extracted using the 2% CTAB method with slight modifications [27].

The ITS regions of rDNA, including ITS1, 5.8S, and ITS2 (ITS) [28]; actin (*ACT*), chitin synthase (*CHS*) [29], glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) partial genes [30]; and Apn2-Mat1-2 intergenic spacer and partial mating type Mat1-2 gene (*ApMat*) [31] of nine selected *Colletotrichum* sp. obtained in this study were amplified and sequenced. The primers used in this study are shown in Table 1.

PCR was performed in 15-μL reaction mixtures containing 0.18 μL of each primer, 0.18 μL of dNTPs, 0.9 U of GoTaq® DNA polymerase (Promega, Madison, WI, USA), and 3 μL of DNA template (20 ng/μL). The PCR protocols were standardized

Table 1. Primer names, primer sequences, and references used in this study.

	Product name	Primer	Direction	Sequence 5′-3′	Reference
ITS	Internal Transcribed Spacer	ITS5	Forward	GGAAGTAAAAGTCGTAACAAGG	White et al. [28]
		ITS4	Reverse	TCCTCCGCTTATTGATATGC	
GADPH	Glyceraldehyde-3-phosphate dehydrogenase	GDF1	Forward	GCCGTCAACGACCCCTTCATTGA	Templeton et al. [32]
		GDR1	Reverse	GGGTGGAGTCGTACTTGAGCATGT	
ACT	Actin	ACT-512F	Forward	ATGTGCAAGGCCGGTTTCGC	Carbone and Kohn [29]
		ACT-783R	Reverse	TACGAGTCCTTCTGGCCCAT	
CHS	Chitin synthase	CHS-79F	Forward	TGGGGCAAGGATGCTTGGAAGAAG	Carbone and Kohn [29]
		CHS-354R	Reverse	TGGAAGAACCATCTGTGAGAGTTG	
ApMAT	Apn2-Mat1-2 Intergenic Spacer and partial mating type Mat1-2 gene	CgDL F6	Forward	AGTGGAGGTGCGGGACGTT	Rojas et al. [31]
		CgMat1 F2	Reverse	TGATGTATCCCGACTACCG	

for each gene as follows: ITS, a first step at 95 °C for 4 min, followed by 35 cycles at 95 °C for 1 min, 58 °C for 1 min and 72 °C for 2 min, and a final step at 72 °C for 10 min; ACT, a first step at 95 °C for 8 min, followed by 35 cycles at 95 °C for 15 s, 61 °C for 20 s, 72 °C for 1 min, and a final step at 72 °C for 5 min; CHS, a first step at 95 °C for 4 min, followed by 35 cycles at 95 °C for 30 s, 58 °C for 30 s, 72 °C for 45 s, and a final step at 72 °C for 7 min; GAPDH, a first step at 94 °C for 5 min, followed by 35 cycles for 94 °C for 1 min, 56 °C for 75 s, and 72 °C for 75 s, and a final step at 72 °C for 10 min; ApMat, a first step at 95 °C for 5 min, followed by 10 cycles at 95 °C for 30 s, 62 °C for 30 s, and 72 °C for 1 min, followed by 35 cycles at 95 °C for 30 s, 52 °C for 30 s, and 72 °C for 1 min, with a final step at 72 °C for 10 min.

Amplified PCR products were cleaned using ExoSAP-IT® (Affymetrix, Santa Clara, CA, USA) and both strands were directly sequenced using BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems™) in a 3130 Genetic Analyzer Sequencer (Applied Biosystems™) at Postgraduate College facilities, Mexico.

2.4. Phylogenetic reconstruction

The DNA sequences obtained from both strands were assembled to obtain consensus sequences for each isolate using BioEdit v7.0.5 [32]. Phylogenetic analysis of the Colletotrichum isolates was performed based on concatenated multi-locus datasets of ITS, ACT, CHS, GAPDH, and ApMAT sequences. Multiple sequences were aligned using the MAFFT v.7 server (http://mafft.cbrc.jp/aligment/server/) under default parameters.

Sequences of Colletotrichum species deposited in the GenBank database belonging to the Musae clade in the C. gloeosporioides species complex [7] were retrieved and included as reference species along with the sequences obtained in this study (Table 2). Concatenated alignment was performed using MESQUITE v.3.10 [33], and Bayesian inference (BI) was performed using four Markov Chains Monte Carlo (MCMC) in Mr. Bayes v.3.2.1 software [34]. Maximum-likelihood analysis was performed in raxmlGUI 1.5b2 [35]. Rapid bootstrapping with 1000 iterations was implemented using the general time reversible model with a gamma distribution.

Nucleotide substitution models were determined using MrModeltest v.2.3 [36] for each gene partition and included in the BI. Two analyses of four MCMC chains were run from random trees for 1,500,000 generations and sampled every 1000 generations. The first 25% of generated trees were discarded as the burn-in phase of each analysis and posterior probabilities were determined for the remaining trees. The phylogenetic tree was rooted with the sequence of C. gloeosporioides sensu stricto CBS 123755. Sequences derived in this study were submitted to GenBank (Table 2). DNA alignment and phylogenetic trees were deposited in TreeBase under ID study S21326.

2.5. Morphology and cultural characteristics

After phylogenetic reconstruction and identification of the pathogen, morphological analysis was performed. Conidia were harvested from the PDA plates, incubated at 28 °C in the dark, and then mounted onto a microscope slide with clear 1% lactic acid. Mycelia conidial and appressorium formation were produced using a slide culture technique [37,38], and 30 of them were selected for measurement using ImageJ software (https://imagej.nih.gov/ij/) through photographs taken by Infinity 1-2 C implemented on an Olympus BX41 microscope (Tokyo, Japan). The shape and size of 50 conidia were measured with the same procedure. Colony characteristics on PDA were recorded after 7 days.

3. Results

3.1. Monoconidial isolates

Original field symptoms observed in 30 avocado fruits collected in the central part of Mexico showed necrotic tissue with small dark brown spots on green-unripe fruit exocarps, which coalesced with age (Figure 1(a)). Twenty-four monoconidial isolates were obtained for further analyses.

Table 2. Isolates of *Colletotrichum* spp. used in this study with GenBank accession numbers.

Species	Strain	Host	Country	GenBank accession numbers				
				ITS	ACT	CHS	GAPDH	ApMat
C. aenigma	ICMP18608	*Persea americana*	Israel	JX010244	JX009443	JX009774	JX010044	KM360143
C. aeschynomenes	ICMP 17673	*Aeschynomene virginica*	USA	JX010176	JX009483	JX009799	JX009930	KM360145
C. alienum	ICMP 12071	*Malus domestica*	New Zealand	JX010251	JX009572	JX009882	JX010028	KC888927
C. asianum	CBS 130418	*Coffea arabica*	Thailand	FJ972612	JX009584	JX009867	JX010053	FR718814
C. fructicola	CPO 27.01	*P. americana*	Mexico	KX268474	KX355488	KX355497	KX687154	KX687163
C. fructicola	CPO 27.02	*P. americana*	Mexico	KX278718	KX355489	KX355498	KX687155	KX687164
C. fructicola	CPO 27.03	*P. americana*	Mexico	KX278719	KX355490	KX355499	KX687156	KX687165
C. fructicola	CPO 27.04	*P. americana*	Mexico	KX278720	KX355491	KX355500	KX687157	KX687166
C. fructicola	CPO 27.05	*P. americana*	Mexico	KX278721	KX355492	KX355501	KX687158	KX687167
C. fructicola	CPO 27.06	*P. americana*	Mexico	KX278722	KX355493	KX355502	KX687159	KX687168
C. fructicola	CPO 27.07	*P. americana*	Mexico	KX278723	KX355494	KX355503	KX687160	KX687169
C. fructicola	CPO 27.08	*P. americana*	Mexico	KX278724	KX355495	KX355504	KX687161	KX687170
C. fructicola	CPO 27.09	*P. americana*	Mexico	KX278725	KX355496	KX355505	KX687162	KX687171
C. fructicola	ICMP 18646	*Tetragastris panamensis*	Panama	JX010173	JX009581	JX009874	JX010032	JQ807839
C. fructicola	CBS 130416	*C. arabica*	Thailand	JX010165	FJ907426	JX009866	JX010033	JQ807838
C. fructicola	GM567 = MTCC 11679	*M. indica*	India	JQ894676	JQ894543	JQ894614	JQ894630	JQ894576
C. gloeosporioides	CBS 112999	*Citrus sinensis*	Italy	JX010152	JX009531	JX009818	JX010056	JQ807843
C. musae	CBS 116870	*Musa* sp.	USA	JX010146	JX009433	JX009896	JX010050	KC888926
C. nupharicola	CBS 470.96	*Nuphar lutea* subsp. *polysepala*	USA	JX010187	JX009437	JX009835	JX009972	JX145319
C. queenslandicum	ICMP 1778	*Carica papaya*	Australia	JX010276	JX009447	JX009899	JX009934	KC888928
C. salsolae	ICMP 19051	*Salsola tragus*	Hungary	JX010242	JX009562	JX009863	JX009916	KC888925
C. siamense	GM018 = MTCC 11672	*M. indica*	India	JQ894653	JQ894533	JQ894608	JQ894624	JQ894556
C. tropicale	ICMP 18653	*Theobroma cacao*	Panama	JX010264	JX009489	JX009870	JX010007	KC790728
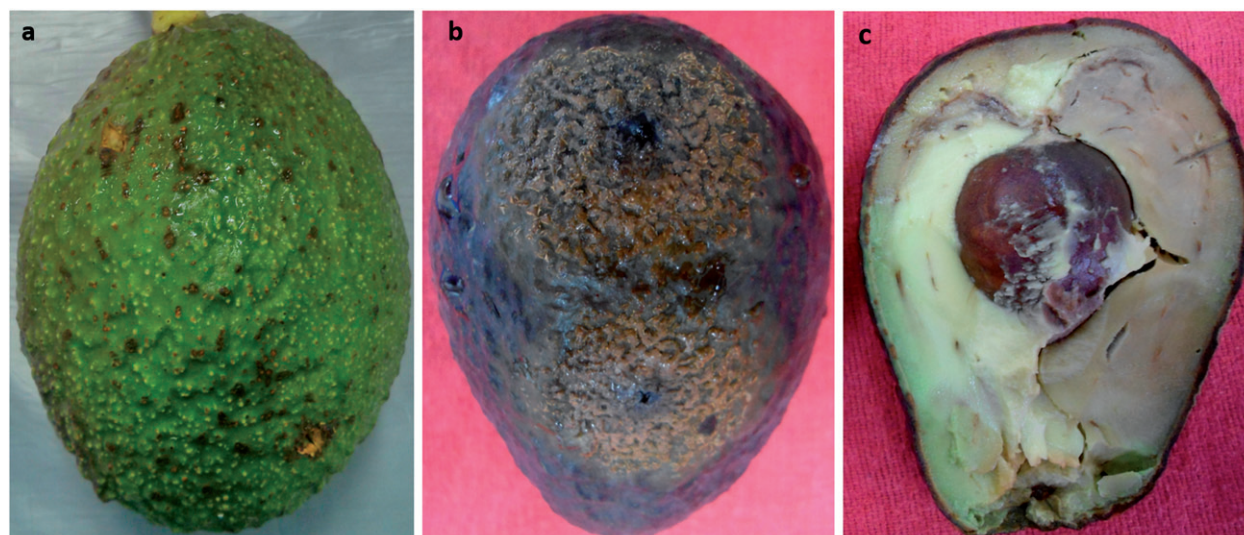
Figure 1. Original anthracnose symptoms (a), symptoms in the pathogenicity test showing abundant sporulation on the pericarp (b) and dark soft rot in the mesocarp (c).

3.2. Pathogenicity test

The pathogenicity test was performed with 24 *Colletotrichum* isolates on unripe fruits of avocado cv. "Hass" to confirm Koch's postulates. The development of small circular necrotic spots was observed at 10 days after inoculation (DAI), and abundant conidia masses were observed on the surface of the avocado fruits for all isolates inoculated (Figure 1(b)). When the fruits were cut, a soft rot with dark brown discoloration in the mesocarp was observed at 10 DAI (Figure 1(c)). Setae were absent. The fungus was re-isolated from the exocarp tissue, as described above, and re-identified morphologically. All of the inoculated isolates were found to be very aggressive because the infection advanced until covering almost 50% of the inoculated fruits.

3.3. Phylogenetic analysis

All isolates were similar in the morphological and pathogenicity tests; thus, nine isolates were selected at random for phylogenetic analysis. The combined gene alignment comprised 23 taxa and 1922 characters, including gaps (ITS: 1–507, ACT: 508–729, CHS: 730–1002, GAPDH: 1003–1266, ApMAT: 1267–1922). The best evolution models of each gene were: K80 + I for ITS, HKY for ACT, SYM + I for CHS, HKY + G for GAPDH, and HKY + I for ApMAT. The consensus tree obtained for Bayesian analysis showed that the nine isolates obtained in this study clustered with the *C. fructicola* clade with a posterior probability of 100%, and differed from the reference isolates. *C. fructicola* was separated from the most closely related species
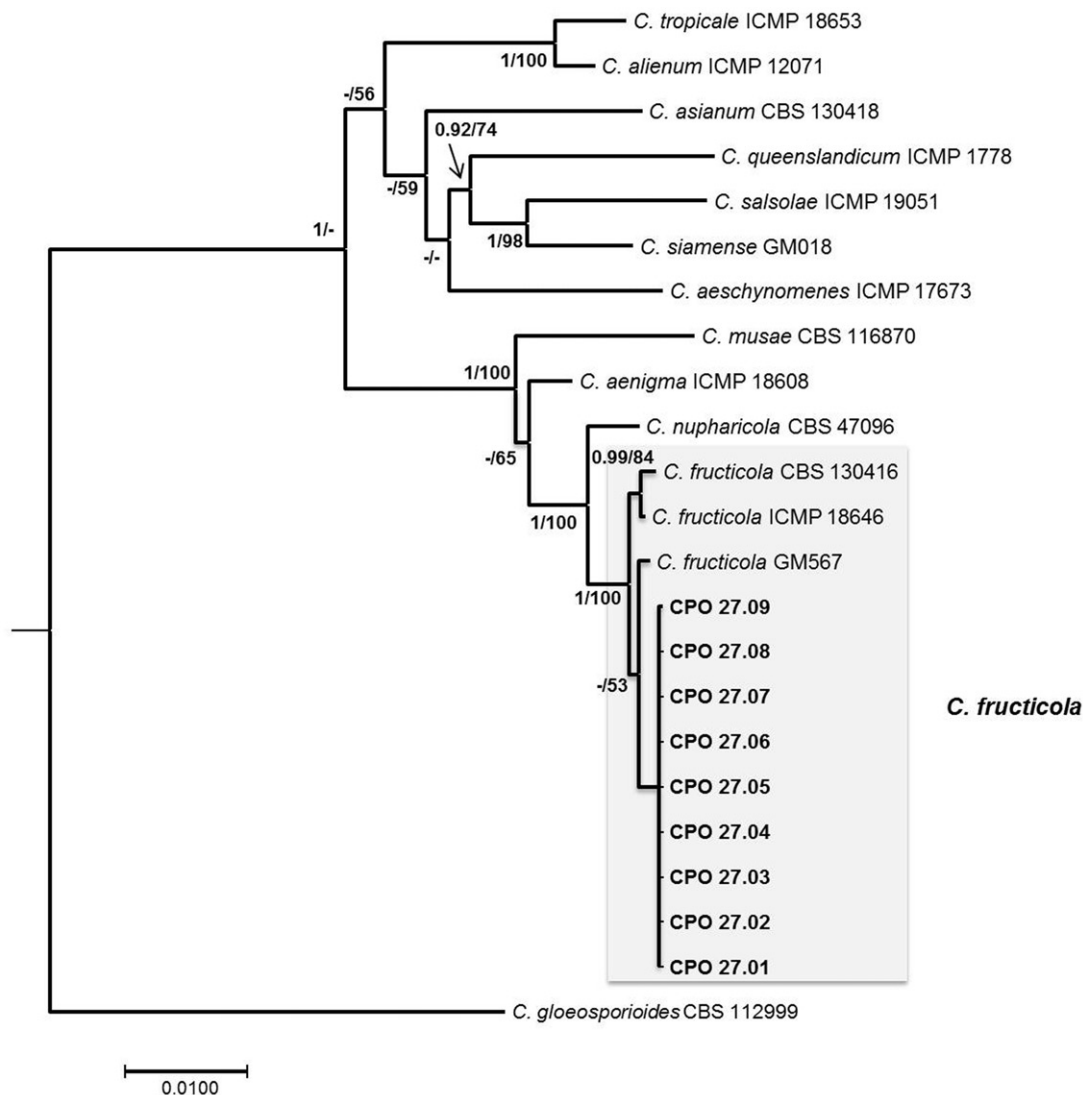
Figure 2. Bayesian consensus phylogenetic tree in the Musae clade of the *C. gloeosporioides* species complex, constructed with concatenated sequences of the ITS region, *ACT*, *CHS*, *GAPDH*, and *ApMat* with the best evolutionary model applied for each gene. Posterior probabilities ≥0.90 and bootstrap support ≥50% are shown in the nodes (BI/ML). Scale bar: substitutions per site.

(*C. nupharicola*) with 100% support (Figure 2). All isolates analyzed in this study by a molecular approach were identical for all genes.

3.4. Morphology and cultural characteristics

Most of the morphological features coincided with those reported by Prihastuti et al. [39]. For all isolates, the growth of colonies on PDA media showed a dark brown color, becoming gray to dark gray at the center with age (Figure 3(a)). The aerial mycelium was gray, dense, with visible conidia masses. Acervuli and setae were absent. The reverse side was grayish with a white halo of mycelium (Figure 3(b)). Conidia were (11.06−) 14 (−16.14) × (3.19−) 4.03 (−4.89) μm (n = 50), one-celled, smooth-walled, hyaline, cylindrical with obtuse to slightly rounded ends; the majority had two rounded guttules (Figure 3(c)). Conidial and mycelial appressoria formed on slide cultures were (7.68−) 8.46

(−9.62) × (5.65−) 6.13 (−6.74) μm (n = 30), brown to dark brown, ovoid for conidial appressoria and slightly obtuse at the tip, pedicellate, and irregular in shape for mycelial appressoria, often containing one or more guttules (Figure 4(a,b)).

4. Discussion

Avocado (*P. americana* Mill.) is one of the most important fruit crops in Mexico because it is the main export product. In 2013, Mexico ranked first worldwide in production and export with 1,467,837 tons and 563,492 tons, respectively [40]; trades forecast that avocado exports for the marketing year 2016–2017 will increase to over 1.0 million metric tons due to expected strong international demand [41].

One of the main problems faced by producers to obtain fruits with high commercial quality is the incidence of fungal diseases. Anthracnose caused by
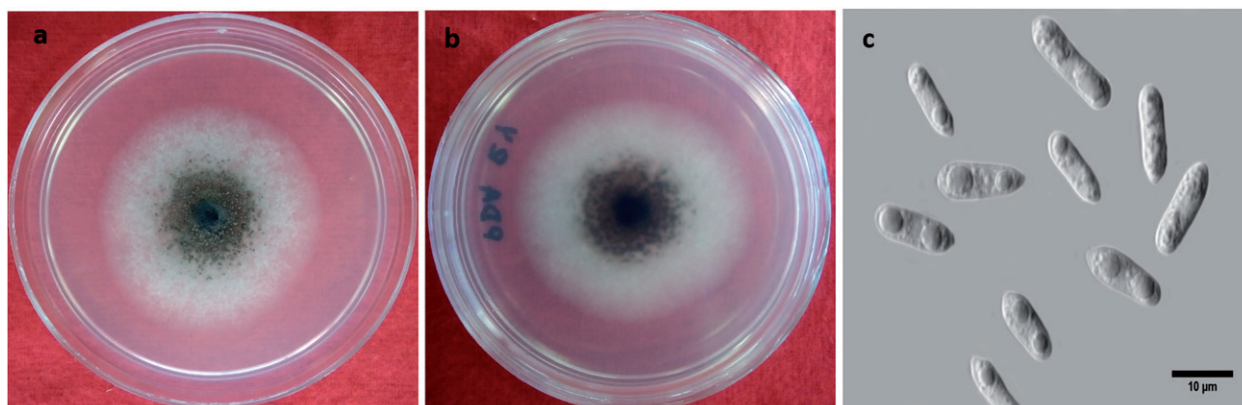
Figure 3. Cultural and morphological characteristics of *C. fructicola*. Culture on PDA medium (a,b), and conidia (c).
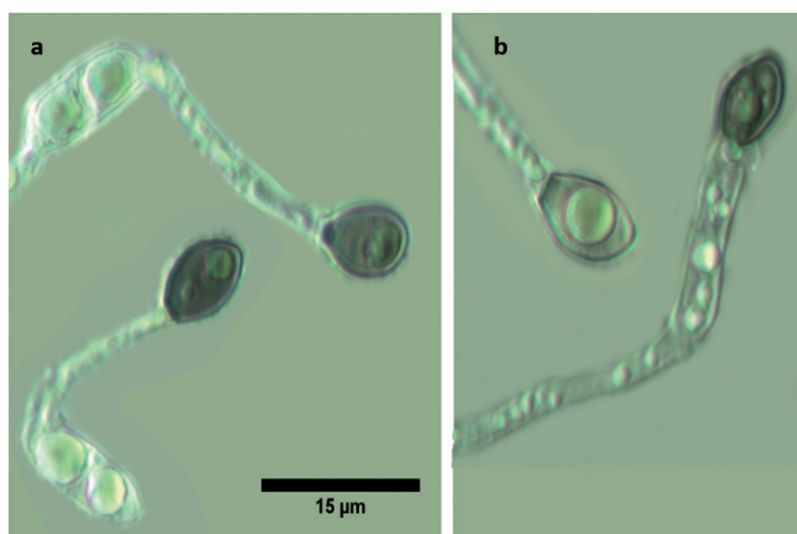

Figure 4. Conidium germlings (a) and mycelia (b) showing formation of pedicellate appressoria. Scale bar of (a) applies for (b).

different species of *Colletotrichum* represents one of the challenges within production systems. Through amplification of ITS sequences, *C. gloeosporioides*, *C. acutatum*, and *C. boninense* were initially described to be associated with avocado anthracnose fruits in the states of Michoacan and Morelos, Mexico, respectively [15,42]. Furthermore, it has been reported that *C. godetiae* and *C. karstii*, which belong to the *C. acutatum* and *C. boninense* complexes, affect avocado in the state of Morelos, Mexico [16,17]. In the present study, *C. fructicola*, a member of the *C. gloeosporioides* species complex, was found to be a causal agent of anthracnose in avocado fruits collected in the state of Hidalgo, in the central part of Mexico, using morphological and multilocus phylogenetic analyses. Nevertheless, variation in colony morphology (colony color, pigment, sporulation, perfect state, and conidia size) has been reported [43] and sterile isolates of *C. fructicola* have been encountered [3], suggesting high genetic variation.

Colletotrichum fructicola was proposed as a crop pathogen for the first time by Prihastuti et al. [39] using a polyphasic approach, affecting coffee berries in Thailand. Since then, it has been reported to cause anthracnose, thus representing an important fungal pathogen in several plantations worldwide [6]. *Colletotrichum fructicola* was the dominant and most aggressive species associated with bitter rot of apple in Uruguay [44] and strawberry in Japan [45]. In China, *C. fructicola* was reported to cause anthracnose of sandy pear (*Pyrus pyrifolia*), an economically significant disease in southern China [43], and was the principal fungus of the *C. gloeosporioides* complex causing citrus anthracnose [46]. Furthermore, *C. gloeosporioides*, previously reported to cause anthracnose in different crops worldwide, was then reclassified. For example, *C. fructicola* is a causal agent of strawberry anthracnose in Korea, which was previously reported as *C. gloeosporioides* [47].

Colletotrichum gloeosporioides sensu stricto as a causal agent of avocado fruits anthracnose has only been reported recently in Turkey [48]. In addition, *C. gloeosporioides sensu stricto* was not present in tropical fruits tested in Laos and Thailand [49]. Diverse studies reported *C. gloeosporioides* as a pathogen causing anthracnose in avocado fruits using analyses of the ITS region, in which the isolates were
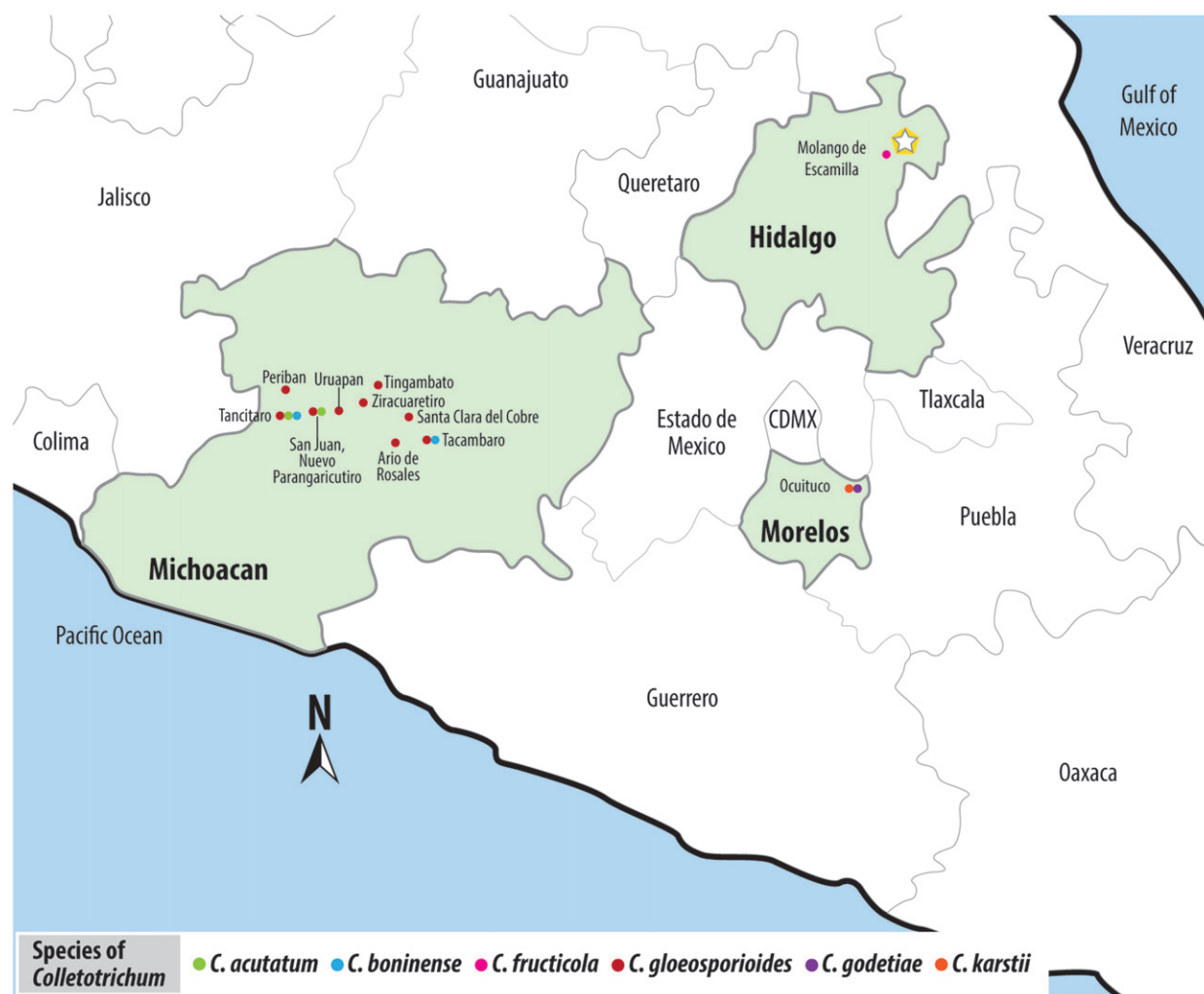
Figure 5. Distribution of the *Colletotrichum* species complex in the main avocado-producing areas in Mexico. Image created by the authors. Map source: INEGI, Mapa Digital de México.

grouped in different clades, suggesting that different species were present [12,15]. Nevertheless, other genes and statistical approaches are needed to determine robust phylogenetic relationships and provide the true species delimitations [23,49]. For example, the intergenic sequence of apn2 and Mat1-2 (*ApMat*) can improve resolution for the definition of the *Colletotrichum* species complex compared to other commonly used loci [2,50], and *ApMat* and *GS* loci can help to resolve species identification in the *C. gloeosporioides* complex affecting *Camellia* [51].

It is possible that *C. fructicola* is amply distributed in other crops and that diverse species can cause the same disease in avocado because of an ample range host and the high diversity of this genus, which have not been previously investigated in Mexico. However, special attention should be given to this species, because even though anthracnose symptoms in the field were not very evident (Figure 1(a)), when the fruits were placed in favorable environmental conditions, the fungus was able to colonize almost the entire fruit in a short time (Figure 1(b)) accompanied by mesocarp soft rot (Figure 1(c)). This observation further indicates the potential aggressiveness of these

isolates and the risk that can be introduced in other areas where *C. fructicola* has not yet been reported to date.

Further research focused on determining the distribution of *Colletotrichum* species is needed for implementation of specific management strategies based on fungicide applications in each avocado production region, because variation in sensitivity can occur as well as the emergence of fungicide-resistant isolates [52,53]. Furthermore, the emergence of more aggressive species of *Colletotrichum* highlights the need for regular surveys, particularly when taking into account the fact that most commercial groves are cv. "Hass" monocultures, the effects of climate change, and the adaptation of pathogens to new varieties and ecological niches.

In conclusion, the present results obtained by molecular and morphological techniques as well as pathogenicity tests suggest that *C. fructicola* is the causal agent of anthracnose and soft rot in avocado fruits cv. "Hass" in the central part of Mexico. This finding is very important because the state of Hidalgo is close to Michoacan, the main avocado-producing state in Mexico, and thus *C. fructicola*

could spread into areas where only *C. gloesporioides*, *C. acutatum*, and *C. boninense* have been reported to date (Figure 5). Moreover, this species should be included in sensitivity to fungicides tests aimed at maintaining the high quality of avocado fruits, which is essential for the exportation trade.

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